IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : John Bertin et al.
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Title : NOVEL MOLECULES OF THE PYRIN/NBS/LRR PROTEIN FAMILY AND USES THEREOF

Art Unit : 1642
Examiner : Karen A. Canella

Commissioner for Patents
P.O. Box 1450
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DECLARATION OF FREDERICK LO UNDER 37 C.F.R §1.132

I, Frederick Lo, declare as follows.

1. I am a Senior Research Scientist at Wyeth. I received an M.S. in Physiology from The Johns Hopkins University in 1994.

2. I supervised a project that evaluated the role of PYRIN-5 (also known as NALP5 and MATER) in the regulation of apoptosis in injured neurons. As detailed below, these studies demonstrated that: (1) PYRIN-5 expression is elevated in injured neurons; (2) PYRIN-5 is expressed in transient focal ischemia; and (3) PYRIN-5 induces neuronal apoptosis.

3. PYRIN-5 EXPRESSION IS ELEVATED IN INJURED NEURONS.

The human PYRIN-5 cDNA sequence was aligned with mouse genomic DNA to identify exons of mouse PYRIN-5. PCR primers were then designed to amplify genomic DNA within a single exon. As a result of the substantial sequence similarity between mouse and rat PYRIN-5 sequences, PCR amplicons were then obtained from rat genomic DNA. The DNA sequences of the rat amplicons were determined and subsequently used to design PCR primers for rat gene expression analysis.

We sought to characterize expression of PYRIN-5 in neuronal cells, as opposed to immune cells of the brain such as microglia. Therefore, the first investigations of PYRIN-5

expression patterns were conducted in cultured rat neurons rather than tissue samples containing multiple cell types.

Cerebellar granule neurons (CGNs) in culture were subjected to injury by transfer to medium lacking serum and containing reduced potassium (K^+). This treatment of serum/K^+ withdrawal induces apoptosis in approximately 60% of cells in 24 hours (Chiang et al. (2001) Proc. Natl. Acad. Sci. USA 98:2814-19). First-strand cDNA was generated from total RNA isolated from the cultured neurons and used as template for PCR amplification with the PYRIN-5-specific primers. PYRIN-5 was found to be weakly expressed in untreated neurons, but showed a substantial elevation of expression in the injured CGNs.

4. PYRIN-5 IS EXPRESSED IN TRANSIENT FOCAL ISCHEMIA.

A portion of the neurodegeneration and brain dysfunction following ischemic stroke results from time-delayed neuronal apoptosis (Li et al. (1995) Stroke 26: 1252-58; Namura et al. (1998) J. Neurosci. 18:3659-68). Following the demonstration that PYRIN-5 gene expression is induced in injured neurons in culture (Paragraph 3, above), similar reverse transcription-PCR investigations were conducted using cortical samples isolated from adult rats subjected to transient middle cerebral artery occlusion (MCAO) followed by a time course of reperfusion. Cortical tissue from a rat receiving no occlusion (sham-operated) as well as contralateral hemispheres of animals at each reperfusion timepoint served as non-injury controls.

PYRIN-5 showed substantially elevated expression in MCAO samples at 1 hour reperfusion (expression was barely detectable at 8 hours). PYRIN-5 mRNA was not detected in tissue from sham-operated animals or in contralateral hemispheres of MCAO rats. This absence contrasts with the weak expression observed in the cultured neurons, possibly resulting from developmental expression or from a cellular stress response to explantation.

5. PYRIN-5 INDUCES NEURONAL APOPTOSIS.

Recombinant expression assays were conducted in cell lines and in primary neurons to evaluate the ability of PYRIN-5 to stimulate apoptosis. The human cDNA sequence of PYRIN-5 was isolated and cloned into a vector providing target protein expression fused to either a myc-His6 epitope or enhanced green fluorescence protein (EGFP).

Expression of PYRIN-5-myc-His6 or PYRIN-5-EGFP resulted in a potent induction of apoptosis in HeLa and NIH-3T3 cells. Programmed cell death induced by PYRIN-5 expression in HeLa was scored by nuclear morphology and activation of fluorogenic caspase-3 substrate. PYRIN-5 induced a significant elevation in apoptosis by both measures.

To determine whether PYRIN-5 also induces apoptosis in neurons, rat CGNs were transfected with a control vector or a vector encoding PYRIN-5. Transfected (EGFP+) neurons were scored for apoptosis by caspase-3 activity or nuclear morphology. Elevated expression of PYRIN-5 resulted in a highly significant increase in apoptosis, with both measures providing equivalent results. The observation that recombinant expression of PYRIN-5 induced neuronal apoptosis suggests that the elevated PYRIN-5 expression observed in injured neurons (Paragraph 3, above) or in MCAO samples (Paragraph 4, above) represents native regulation of the activities of the molecule in neuronal death.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

May 6, 2005
Date

Frederick Lo, M.S.